T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR


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Regulatory T (Treg) cells safeguard against autoimmunity and immune pathology. Because determinants of the Treg cell fate are not completely understood, we have delineated signaling events that control the de novo expression of Foxp3 in naive peripheral CD4 T cells and in thymocytes. We report that premature termination of TCR signaling and inhibition of phosphatidylinositol 3-kinase (PI3K) p110α, p110δ, protein kinase B (Akt), or mammalian target of rapamycin (mTOR) conferred Foxp3 expression and Treg-like gene expression profiles. Conversely, continued TCR signaling and constitutive PI3K/Akt/mTOR activity antagonised Foxp3 induction. At the chromatin level, di- and trimethylation of lysine 4 of histone H3 (H3K4me2 and -3) near the Foxp3 transcription start site (TSS) and within the 5′ untranslated region (UTR) preceded active Foxp3 expression and, like Foxp3 inducibility, was lost upon continued TCR stimulation. These data demonstrate that the PI3K/Akt/mTOR signaling network regulates Foxp3 expression.

Specialized cell types in multicellular organisms are defined by distinct patterns of gene expression (1). During their differentiation from hematopoietic stem cells, developing T cells undergo progressive restriction of their lineage potential. After the CD4/CD8 lineage choice in the thymus, CD4 lineage cells remain able to adopt a naive or regulatory cell fate, and naive CD4 T cells can opt for a range of Th lineages or, alternatively, become regulatory T (Treg) cells after activation (2, 3). The choice of Th lineage is important for effective immune responses to specific pathogens, and the balance between effector and regulatory cells is critical to ensure immune competence while avoiding immune pathology and autoimmunity. Thymus-derived Treg cells are generated via a TGFβ independent pathway that requires costimulatory signals (2–4) and typically expresses the CD4 T cell receptor signaling controls Foxp3 expression in activated CD4 T cells. Naive CD42L1bCD4+CD25− LN T cells were isolated by flow cytometry and labeled with CFSE. Residual Foxp3 expression was minimal as judged by intracellular staining (Fig. 1a, post sort) and remained unchanged after 18 h of activation with plate bound anti-TCR and anti-CD28 (Fig. 1a, 18th anti-TCR, anti CD28) and after another 36 h with anti-TCR (Fig. 1a and d, with TCR signaling). However, Foxp3 RNA and protein were markedly up-regulated when the same cells were activated for 18 h with plate bound anti-TCR and anti-CD28 and then maintained without TCR stimulation for 36 h (Fig. 1a and d, no TCR signaling). Hence, the continued availability of TCR signals appeared to control Foxp3 expression in newly activated CD4 T cells. TCR/CD28 engagement triggers multiple signaling pathways (21). To investigate which of these control Foxp3 expression, we screened small molecule inhibitors of enzymes involved in signal transduction. No increase in Foxp3 expression was seen when inhibitors of calcineurin/NTF (cyclosporin A and FK-506), mitogen activated kinases (SB203580, PD98059), protein kinase-C (UCN-1028c, calphostin C, Myr-N-FARKGALRQ-NH2, G6976, Ro-32-0432, Ro-31-8220), glycochen synapse kinase-3 (SB21673), PPARγ (GW501516), and γ-secretase/Notch (L-685458; data not shown) were added to 18 h activated CD4 T cells. By contrast, the PI3K inhibitor LY294002 potently induced Foxp3 in this assay (Fig. 1a–c, L, LY). Rapamycin, an inhibitor of the protein kinase mTOR, which lies in the same signaling pathway (25), also induced Foxp3 (Fig. 1a–c, rapa, L). The combination of LY294002 and rapamycin induced Foxp3 in >75% of CD4 T cells (Fig. 1a–c, rapa +LY) and synergized with TGFβ, resulting in >90% Foxp3 induction in the absence of exogenous cytokines (Fig. 1a–c, TGFβ + rapa + LY). CFSE labeling ruled out the selective expansion of preexisting Foxp3+ cells (Fig. 1a), and cell counts showed a substantial net increase in Foxp3+ cell numbers (Fig. 1c). De novo induction of Foxp3 by PI3K and mTOR inhibitors was formally demonstrated by using AND TCR transgenic Rag1−/−CD62L1bCD4+CD25− LN T cells, which are devoid of preexisting Foxp3+ cells [Fig. 1d and supporting information (SI) Fig. 1d].

Results

Premature Withdrawal of TCR Signals and Inhibitors of the PI3K/mTOR Pathway Induce Foxp3 Expression in Activated CD4 T Cells. Naive CD42L1bCD4+CD25− LN T cells were isolated by flow cytometry and labeled with CFSE. Residual Foxp3 expression was minimal as judged by intracellular staining (Fig. 1a, post sort) and remained unchanged after 18 h of activation with plate bound anti-TCR and anti-CD28 (Fig. 1a, 18th anti-TCR, anti CD28) and after another 36 h with anti-TCR (Fig. 1a and d, with TCR signaling). However, Foxp3 RNA and protein were markedly up-regulated when the same cells were activated for 18 h with plate bound anti-TCR and anti-CD28 and then maintained without TCR stimulation for 36 h (Fig. 1a and d, no TCR signaling). Hence, the continued availability of TCR signals appeared to control Foxp3 expression in newly activated CD4 T cells. TCR/CD28 engagement triggers multiple signaling pathways (21). To investigate which of these control Foxp3 expression, we screened small molecule inhibitors of enzymes involved in signal transduction. No increase in Foxp3 expression was seen when inhibitors of calcineurin/NTF (cyclosporin A and FK-506), mitogen activated kinases (SB203580, PD98059), protein kinase-C (UCN-1028c, calphostin C, Myr-N-FARKGALRQ-NH2, G6976, Ro-32-0432, Ro-31-8220), glycochen synapse kinase-3 (SB21673), PPARγ (GW501516), and γ-secretase/Notch (L-685458; data not shown) were added to 18 h activated CD4 T cells. By contrast, the PI3K inhibitor LY294002 potently induced Foxp3 in this assay (Fig. 1a–c, L, LY). Rapamycin, an inhibitor of the protein kinase mTOR, which lies in the same signaling pathway (25), also induced Foxp3 (Fig. 1a–c, rapa, L). The combination of LY294002 and rapamycin induced Foxp3 in >75% of CD4 T cells (Fig. 1a–c, rapa +LY) and synergized with TGFβ, resulting in >90% Foxp3 induction in the absence of exogenous cytokines (Fig. 1a–c, TGFβ + rapa + LY). CFSE labeling ruled out the selective expansion of preexisting Foxp3+ cells (Fig. 1a), and cell counts showed a substantial net increase in Foxp3+ cell numbers (Fig. 1c). De novo induction of Foxp3 by PI3K and mTOR inhibitors was formally demonstrated by using AND TCR transgenic Rag1−/−CD62L1bCD4+CD25− LN T cells, which are devoid of preexisting Foxp3+ cells [Fig. 1d and supporting information (SI) Fig. 1d].


The authors declare no conflict of interest.

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Data deposition: Gene expression data have been deposited in the ArrayExpress database, www.ebi.ac.uk/ arrayexpress (accession no. E-MIRR-1241).

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Activation with peptide-pulsed antigen presenting cells also conferred competence for Foxp3 induction by PI3K inhibitors (Fig. S1b).

TCR/CD28 activation followed by PI3K/mTOR inhibition induced Foxp3 not only in peripheral CD4 T cells, but also in CD4⁺CD8⁻CD25⁻ thymocytes (Fig. S1c).

Differential Role of p110 Isoenzymes in Foxp3 Regulation. Selective PI3K isozenzyme inhibitors have recently been characterized at the biochemical, structural, and biological level (22–25) (Table S1). We utilized such compounds to define the role of specific PI3K catalytic subunits in Foxp3 regulation. TGX115 did not affect Foxp3 expression at concentrations that selectively inhibit p110β (0.1 μM; 23), indicating that p110β does not control Foxp3 in this setting. Similarly, the p110γ inhibitor AS-605240 induced Foxp3 expression only at concentrations far in excess of those required to inhibit p110γ (0.008 μM; ref. 22 and Fig. 1f).

IC-87114 is highly selective for p110δ (23) and induced Foxp3 in 10–15% more cells than TCR deprivation alone, demonstrating a contribution of p110δ to the regulation of Foxp3. PI-103 strongly induced Foxp3 at concentrations below its in vitro IC₅₀ for mTOR (0.02 μM) and around the in vitro IC₅₀ for p110α (0.008 μM) (23). PIK90 strongly induced Foxp3 at 0.1 μM (Fig. 3a), where it inhibits p110α (and p110γ, which did not induce Foxp3; see above). Higher concentrations of PI-103 and PIK90 affect p110α (23), but Foxp3 induction by PI-103 and PIK90 exceeded that of full p110δ inhibition by IC-87114 (Fig. 1f). These data identify p110α as a dominant isozenzyme and the hierarchy of p110α > δ > β/γ may set Foxp3 regulation apart from lymphocyte activation, where p110δ appears dominant (25, 26).

TCR Signaling and the PI3K/Akt/mTOR Network. Protein kinases of the Akt/PKB family are components of the PI3K/Akt/mTOR...
network, and their activity is regulated by PI3K via PDK1 and by one of the two known mTOR-containing complexes, mTORC2 (27). We treated activated CD4 T cells with the allosteric Akt inhibitor Akti-1/2 (28) and found Foxp3 induction at concentrations around its IC50 for Akt1 and Akt2 (58 nM and 210 nM, respectively). This shows that inhibition of Akt and PI3K and mTOR can drive Foxp3 induction (Fig. 1g).

T cell activation results in the sustained activation of the PI3K/Akt/mTOR network (21), reflected in the phosphorylation of S6 ribosomal protein (pS6), a direct target of the mTOR-regulated p70 S6 kinase S6K1 (27). Intracellular staining showed that TCR/CD28 signaling (but not anti-CD28 alone) induced and maintained high levels of pS6 (Fig. 2a). Upon withdrawal of TCR antibody from 18 h activated T cells, S6 phosphorylation declined only gradually (Fig. 2b), correlating with their ability to enhance Foxp3 induction.

Constitutive Activation of the PI3K/Akt/mTOR Network Antagonises Foxp3 Induction. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is the major negative regulator of the PI3K/Akt signaling pathway, and its loss results in constitutive Akt activity (29). To test the concept that PI3K/Akt/mTOR signaling controls Foxp3, we compared the inducibility of Foxp3 in TCR/CD28 activated PTEN-deficient and control T cells (29) in response to the classical Foxp3 inducer TGFβ versus PI3K inhibitors. The frequency of TGFβ-induced Foxp3 cells was considerably lower in PTEN-deficient than in control CD4 cells, but the PI3K inhibitor LY294002 restored Foxp3 induction in PTEN-deficient CD4 T cells (Fig. 2c). These data add genetic evidence that PI3K/Akt/mTOR signaling controls Foxp3 expression in activated T cells.

PI3K/mTOR Inhibitors Induce Treg-Like mRNA and microRNA Expression Profiles. To address whether Foxp3 was induced in isolation or as part of a Treg-like transcriptional program, we performed cDNA expression arrays 24 h after PI3K/mTOR inhibition. Comparison with control activated T cells showed that, in addition to Foxp3, numerous Treg cell markers were up-regulated, including IL2ra (3.0x), IL2rb (3.0x), and CItla4 (2.9x) and members of the suppressor of cytokine signaling (Socs) family Socs1 (3.1x), Socs2 (8.3x), and Socs3 (10.5x). As expected from a Treg-like program, the lymphokine transcripts Il2, Ifng and Il3 were strongly down-regulated (112x, 56x, and 7.8x, respectively). Next, we compared PI3K/mTOR inhibitor-induced cells and freshly isolated Treg cells with naive CD4 T cells and found substantial coregulation: More than half of the transcripts up-regulated in Treg cells were also up-regulated in Foxp3-induced cells (775 of 1376, 56%). Even more strikingly, 87% (1,243 of 1,431) of transcripts that were down-regulated in Treg cells were also down-regulated in response to PI3K/mTOR inhibition (Fig. 3a). Functional annotation showed that up-regulated tran-

Fig. 2. TCR signaling controls the expression of Foxp3 via PI3K/mTOR/Akt. (a) Naive LN CD4 T cells were stimulated with anti-TCR and anti-CD28 for 1 or 18 h or for 1 h with anti-CD28 alone (no TCR signaling). S6 phosphorylation was determined by intracellular staining. (b) Naive LN cells were activated as in a. After 18 h, rapamycin (25 nM) or LY294002 (10 μM) were added for 1 h, or the cells were cultured for the indicated time in the absence of anti-TCR. pS6 levels were determined as in a. (Inset) Immunoblotting confirmed declining pS6 in response to TCR signal deprivation, rapamycin, and LY294002. (c) Top) PTEN-deficient and control CD4 T cells were depleted of preexisting Treg cells and activated with anti-TCR/CD28. (Middle) TGFβ (0.3 ng/ml) was added, and Foxp3 expression was assessed 48 h later. (Bottom) As a control, the PI3K inhibitor LY294002 was added and cells were deprived of TCR signaling after 18 h of activation.

a

b

Fig. 3. PI3K/mTOR inhibition initiates a Treg-like transcriptional program in newly activated T cells. (a) Gene expression differences between Treg cells versus naive CD4 T cells and in 24 h PI3K/mTOR inhibitor-treated cells versus naive CD4 T cells. Shown are the numbers of up- and down-regulated transcripts and the intersection of expression differences between Treg and Foxp3 induced cells. (b) Functional annotation of coregulated transcripts in Treg cells and Foxp3 induced cells relative to naive CD4 T cells, using DAVID. (c) microRNA expression by Treg cells and Foxp3 induced cells compared by qPCR. mir-142-3p and mir-21 are shown as examples for Treg-like and non-Treg-like microRNA expression by Foxp3 induced cells. The expression profiles of eight other microRNAs are listed.

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scripts were enriched for processes of cellular, macromolecular and primary metabolism, cell division, and cell cycle and for the functional terms nucleotide binding, electron transporter, and kinase regulatory activity. Down-regulated transcripts represented distinct processes, in particular transcriptional regulation. Only a minority of genes that were coregulated in ex vivo Treg cells and Foxp3 induced cells were known genomic targets of Foxp3 (Fig. S2). MicroRNAs are important mediators of posttranscriptional gene regulation and naïve CD4 T cells and Treg cells express distinct microRNAs (31). Of the 10 microRNAs we profiled, 7 showed Treg-like expression in Foxp3-induced cells (Fig. 3c).

Taken together, our analysis suggests that PI3K/mTOR signaling controls not only Foxp3 and its direct targets, but a wider Treg-like transcriptional program (30).

**No Detectable Involvement of TGFβ in Foxp3 Induction by PI3K and mTOR Inhibitors.** Because TGFβ is a powerful inducer of Foxp3 expression (14–17) and synergizes with PI3K/mTOR inhibitors (Fig. 1), we addressed its requirement in this system. TGFβ binding induces phosphorylation of receptor-associated Smad2 and Smad3, providing a sensitive indicator of TGFβ signaling. pSmad2 (S465/467) was readily detectable in cells exposed to TGFβ (Fig. 4a, lane 1) but not in cells subjected to TCR signal deprivation (Fig. 4a, lane 2) or PI3K and mTOR inhibition (Fig. 4a, lanes 3 and 4). Neutralizing TGFβ antibodies and the Smad kinase inhibitor SB 431542 (32) blocked Foxp3 induction by TGFβ, but did not affect Foxp3 induction by PI3K/mTOR inhibitors (Fig. 4b). Hence, TGFβ appears dispensable for Foxp3 induction by TCR signal deprivation and PI3K/mTOR inhibition.

**Histone Modifications Mark a Window of Opportunity for Foxp3 Induction by PI3K and mTOR Inhibition.** T cell activation was required for Foxp3 induction, and Foxp3 inducibility was maximal in T cells activated for 18 h before PI3K/mTOR inhibition. Earlier addition of inhibitors blocked activation (ref. 19 and data not shown), and Foxp3 induction was inefficient at later time points (Fig. 5 Top Left). Hence, the competence for Foxp3 expression induced by activation of CD4 T cells is transient and continued TCR signaling antagonizes Foxp3 inducibility.

The expression of the Foxp3 locus is intimately linked to its chromatin structure (33, 34). Permissive posttranslational histone modifications are found in Treg cells at the Foxp3 promoter, the intronic differentially methylated region 3 (DMR3), and the recently described +2079 to +2198 enhancer (33–35). To explore how continued TCR signaling reduces the competence of CD4 T cells to express Foxp3, we considered that chromatin marks can provide important information not only about the actual expression, but also the potential for the expression of developmentally regulated loci (36). We used ChiP (chromatin immunoprecipitation) to analyze histone modifications at the Foxp3 locus in male (XY) cells (Foxp3 is X-linked). We compared CD4 cells activated for 18 h (high potential for Foxp3 induction, no Foxp3 expression) to the same cells after 72 h of TCR stimulation (reduced potential for Foxp3 induction, no Foxp3 expression) and CD4 cells activated for 18 h and then exposed to PI3K/mTOR inhibitors (high Foxp3 expression). Oct4, which is silent in T cells, and the actively transcribed Ikaros locus served as controls (Fig. 5). Interestingly, H3K4 di- and trimethylation was found near the Foxp3 TSS (34) and the 5' UTR not only in Foxp3+ cells but also in 18-h activated CD4 T cells, which had the potential for Foxp3 induction but did not actually express Foxp3. In contrast, H3K4me2 and -3 were lost after 72 h of continuous TCR signaling (Fig. 5). These data
link H3K4 methylation to the potential for Foxp3 expression. Conversely, the TCR signaling-induced loss of this permissive chromatin mark correlates with declining competence for Foxp3 expression.

**Discussion**

We have shown that TCR signaling via PI3K p110α, p110δ, Akt, and mTOR controls Foxp3 expression in activated CD4 lineage thymocytes and peripheral T cells. Inhibition of this signaling network conferred de novo expression of Foxp3 and Treg-like mRNA and miRNA profiles. Conversely, constitutive activation of the PI3K/Akt/mTOR network in PTEN-deficient T cells reduced Foxp3 inducibility, which was restored by PI3K inhibition. Foxp3 induction by TCR signal deprivation and PI3K/mTOR inhibition shares features with the development of natural Treg cells. TGFβ3, although synergistic, is not required for the induction of Foxp3 expression in the thymus (8, 9) or in response to TCR signal deprivation and PI3K and mTOR inhibition (this article), and both are markedly enhanced by costimulatory signals (refs. 2 and 3 and Fig. S3). Whereas CD28 signals can interfere with adaptive Treg differentiation driven by TGFβ3 (e.g., 13, 17), cooperation between TGFβ3 and CD28 was reported for the Foxp3 enhancer element at +2079 to +2198 (35).

Evidence for an involvement of the PI3K/Akt/mTOR network in Treg differentiation and function has been accumulating: Treg cell expression under review indicate that Akt signaling interferes with Foxp3 (18–20), and exciting data published while this manuscript was in press suggest that Akt signaling interferes with Foxp3 expression in vivo (in vitro and in vivo (38). Our data provide a rationale for these genetic and pharmacological data by demonstrating that (i) TCR signaling controls Foxp3 expression via a signaling network with the key components PI3Kα and δ, Akt, and mTOR, the mammalian target of rapamycin, and (ii) the timing of PI3K/Akt/mTOR inhibition relative to TCR signaling is critical for the outcome. Interestingly, Akt signaling appears to be compromised in a PTEN-dependent fashion in established Treg cells (39).

Directing cell fate choice in the immune system by pharmacological means is potentially attractive, and a growing number of suitable compounds are approved for clinical use. The ability of small molecule inhibitors to block PI3K/mTOR/Akt signaling temporarily rather than permanently may be beneficial because constitutive p110δ deficiency promotes the differentiation of Treg cells in the thymus, but impairs their subsequent maintenance in the periphery (26, 27).

In our experiments, the duration of TCR signaling affects the probability of Foxp3 induction. Weak TCR signals and limited costimulation had been linked to Foxp3 induction (13, 17, 40). We find higher Foxp3 induction rates by optimal anti-TCR/CD28 signaling followed by TCR signal deprivation and PI3K/mTOR inhibition, consistent with a recent two-stage model of Treg differentiation in which TCR signals are required for the up-regulation of genes like Cd25 that predispose to Treg differentiation, but not for the subsequent induction of Foxp3, which occurs in the absence of additional TCR signals (41). Treg development can result from the recognition of tissue-specific antigens expressed by thymic medullary epithelium (42). Such antigens are expressed in a mosaic fashion (43) and may therefore induce transient rather than sustained TCR signals, which in turn could contribute to the outcome of Foxp3 induction versus negative selection. Our data further show that continued TCR signaling extinguishes the competence for Foxp3 induction in naive CD4 T cells. ChIP analysis identified Foxp3 promoter 10 kbp 5′ and 3′ to the Foxp3 TSS and within the 5′ UTR as a mark for the inducibility of Foxp3, because these permissive histone modifications were induced by the activation of naive CD4 T cells and persisted upon the induction of Foxp3 expression, but declined in parallel with the loss of Foxp3 inducibility in response to continued TCR signaling. The challenge now is to understand how signaling, transcription factors, and chromatin components cooperate to translate the duration of TCR signaling into a determinant of Treg cell fate choice.

**Materials and Methods**

**Cell Sorting and Culture.** Animal work was done under the Animals (Scientific Procedures) Act, U.K. Lymph node (LN) cells or thymocytes from wild-type (C57BL/6, BALB/c, or C57BL/6 × 129) and TCR transgenic (44) Rag1−/− or IckCre Ptenflaxial mice (29) were sorted by flow cytometry as described in ref. 31. LN CD4+ CD25− CD62Lhi T cells or CD4+ CD8+ CD25− thymocytes were actived with plate bound anti-TCR (H57; 200 ng/ml; Pharmingen) and anti-CD28 (2 μg/ml; Pharmingen). AND TCR transgenic cells were also activated with B10.BR antigen presenting cells and pigeon cytochrome-C peptide 81–104 (44). Intra- cellular staining for Foxp3 and pTEFb was done as advised by the manufacturers (eBiosciences). Anti-pS6 Ser-235/236 (Jackson ImmunoResearch).

**RT-PCR, Northern Blot Analysis, and Immunoblotting.** Total RNA was isolated by using RNA-Beet (Tel-Test) and reverse transcribed. Real-time PCR analysis was normalized to the geometric mean of Ywhaz and Ube2L3 as described in ref. 31. The following primer sequences were used: Ywhaz, CTGGTCTGAGCCGCCTTGATTGCAT (forward), TCCTGTGCGCAGAATGGTGGG (reverse); Ube2L3, AGGAGCTGTAAGAGGAGGCTTGA (forward), TGGTGGATGATGACTCTGCTGGA (reverse); and Foxp3, ACTGGCATTGGCCTACTCTCCAG (forward); CGCGATGAGCTCCTTCCAGTGTT (reverse). Immunoblotting was performed as described in ref. 31. Quantitative real-time RT-PCR of miRNAs used gene specific RT primers and TaqMan MicroRNA Assay Mix (Applied Biosystems) as described in ref. 31.

**Chromatin Immunoprecipitation.** ChIP was done as described in ref. 45, using 20 μg of chromatin mixed with 80 μg of Drosophila S2 chromatin with 2.5 μl of anti-H3K4me2 (Upstate; catalog no. 07-030), anti-H3K4me3 (Abcam; catalog no. ab8580-100), or rabbit anti mouse IgG (Dako Cytomation; catalog no. A0082). The following primer sequences (5′ to 3′) to Foxp3 292 – 298 to 1055 (TSS; numbering according to ref. 34) CATTGAATCCCTCTTCCTGGTGT (forward), GTTGTGTCTGATAAGTGGG (reverse); Foxp3, 5′ UTR + 4550 to + 4621 (DMR3) GGTGTCACAGAAGCGCGCAG (forward), CAGCTCTGTA-GGGGGTTCTC (reverse); Foxp3, 5′ UTR + 6144 to + 6280 (exon-1) CAATTCTCCTGACTCTGCTTCCA (forward); GGAAGCTGTCATGGGAAATGACTCTCTGTTGATGGG (reverse); and Foxp3, 5′ UTR + 4754 for TGCAGCCCCTCAGAGAGGCGGAAAAGCCCACTAGG (reverse).

**Gene Expression Arrays and Bioinformatic Analysis.** Affymetrix mouse genome 430 2.0 arrays were used in duplicate. Analysis was done in Bioconductor, using robust multi array normalization (www.bioconductor.org). Individual P values were corrected according to Benjamini and Hochberg (46). Only transcripts with both an adjusted P value <0.05 and a fold-change of >1.5 were considered differentially expressed. Functional annotation used DAVID (http://david.abcc.ncifcrf.gov).

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